

Interaction of CD31 with a Heterophilic Counterreceptor Involved in Downregulation of Human T Cell Responses

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Summary

CD31 is a 130-kD glycoprotein of the immunoglobulin (Ig) superfamily expressed on the surface of endothelial cells, platelets, and several leukocyte subsets. Previous reports indicated that CD31 can mediate intercellular adhesion via both homophilic and heterophilic interaction mechanisms. Using a soluble recombinant CD31-Ig fusion protein (CD31 receptor globulin [Rg]), we demonstrate here that human CD31⁻ T lymphocytes and CD4⁺CD31⁻ T cell clones express a heterophilic CD31 ligand that is upregulated 18 h after activation. Interaction of CD31Rg with CD31⁻ T helper cell (Th) clones was divalent cation independent but could be blocked by heparin, thus indicating that the CD31 counterreceptor on T cells can be distinguished from the ligands identified on other cell types. Moreover, a single chain protein of 120 kD was precipitated by CD31Rg from the lysates of CD31⁻ Th clones. CD31Rg completely downregulated the proliferative response and cytokine production (interleukin-4, interferon- γ , and tumor necrosis factor- α) of CD31⁻ Th clones when the cells were maximally stimulated via immobilized CD3 monoclonal antibody. These results suggest that interaction of CD31 with a heterophilic counterreceptor on T lymphocytes can interfere with a positive regulatory pathway of T cell activation, or directly signal T cells to downregulate immune function.

Regulation of the various functions of immune cells requires the coordinate action of soluble as well as surface-bound molecular mediators. The interaction of cell surface adhesion molecules with their cognate ligands on adjacent cells or components of the extracellular matrix thereby facilitates the correct juxtapositioning of cells involved in communication with one another. Simultaneously, engagement of these surface glycoproteins often results in transduction of cellular signals that direct specific effector functions.

One recently characterized member of the Ig superfamily of cell surface proteins, CD31, also known as platelet-endothelial cell adhesion molecule 1 (PECAM-1), has been proposed to function as such a cell adhesion molecule (1). CD31 is a 130-kD glycoprotein expressed on a variety of cell types of the vascular compartment, i.e., endothelial cells, platelets, monocytes, and granulocytes (2–4). Within the population of T lymphocytes, CD31 is expressed on a subset of cells that is predominantly associated with the CD8⁺CD45RA⁺ phenotype (5). Molecular cloning and analysis of the predicted protein sequence revealed that the extracellular domain of CD31 consists of six C2-type Ig homology repeats with structural similarity to Fc γ R and adhesion molecules such as carcinoembryonic antigen and neuronal cell adhesion molecule (3, 4, 6).

Although the full range of physiological functions of CD31 has not been established as yet, a number of lines of evidence suggest that it participates in cell–cell interaction events. On endothelial cells, CD31 is predominantly sequestered at sites of intercellular contact (7), and mouse L cells transfected with CD31 cDNA aggregate in a CD31-dependent manner (8, 9). The mechanism whereby CD31 mediates cellular adhesion appears to be complex; CD31 has been demonstrated to bind to CD31 in homophilic interactions, as well as to participate in heterophilic interactions involving glycosaminoglycans (GAGs)¹ on the surface of adjacent cells (8–11). In addition to its mere adhesive function, CD31 is considered to control dynamic processes such as cell activation and migration. Coligation of CD31 and Fc γ Rs on monocytes has been shown to induce oxidative responses and release of inflammatory cytokines (4, 12). On T lymphocytes and myeloid cell types, ligation of CD31 by mAbs upregulates the adhesive functions of β_1 and β_2 integrins, respectively (13, 14). Abs to CD31 interfere with the transendothelial migration of neutrophils and monocytes, both in vitro and in vivo (15–18), indicating

¹Abbreviations used in this paper: GAG, glycosaminoglycan; Rg, receptor globulin; RT, reverse transcriptase; T-CD31L, T cell CD31 ligand.

that CD31 plays a crucial role in the recruitment of leukocytes to sites of inflammation.

Recent evidence also suggests that CD31 expressed on the surface of leukocytes may directly participate in the development of T cell-mediated immune responses. Within the population of human CD4⁺ T lymphocytes, CD31 expression appears to differentiate between two functionally distinct subsets (19). CD4⁺CD31⁺ cells have been reported to exert suppressor inducer activity for B cell IgG synthesis, whereas the majority of helper activity for IgG production by B cells was found within the CD4⁺CD31⁻ population. Furthermore, a CD31 mAb was able to block T cell suppressor function in a B cell IgG synthesis system (19), and recently a peptide derived from the sixth Ig-like domain of CD31 was found to inhibit MLRs (20). Considering the restricted expression of CD31 on distinct T lymphocyte subsets, we supposed that these functional effects may be related to a heterophilic interaction of CD31 expressed on T cells and/or accessory cells with the surface of CD31⁻ T lymphocytes.

To characterize this putative heterophilic counterreceptor for CD31 on human T lymphocytes, we constructed a soluble recombinant CD31 receptor globulin (CD31Rg) consisting of the entire extracellular part of CD31 joined to the constant region of human IgG1. We provide phenotypic, biochemical, and functional evidence that activated human T lymphocytes and T cell clones express a novel heterophilic ligand for CD31, which is involved in the downregulation of Th cell responses after antigen receptor/CD3 triggering.

Materials and Methods

Abs. Mouse mAb 7E4 (IgG2a) to CD31 (4) and 4H1-A7 to canine adenovirus 2 (IgG2a control) were prepared by Dr. O. Majdic at our Institute. mAbs MEM-57 (IgG2a) to CD3 and AFP-01 to α -fetoprotein (IgG1 control) were kindly provided by Dr. V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). mAb 5E6 (IgG1) to CD31 was obtained from The 4th International Workshop on Human Leukocyte Differentiation Antigens. Human IgG1 was purchased from Sigma Chemical Co. (St. Louis, MO). mAbs OKT3 to CD3 and anti-Leu28 to CD28 were obtained from Ortho Pharmaceuticals (Raritan, NJ) and Becton Dickinson & Co. (Mountain View, CA), respectively.

Construction and Expression of Soluble Recombinant Rgs. A CDM8-derived IgG1 expression plasmid encoding CD2Rg, constructed essentially as described (21), was kindly provided by Dr. B. Seed (Massachusetts General Hospital, Boston, MA). CD31Rg and M6Rg were constructed in the IgG1 expression plasmid by replacing the extracellular domain sequences of CD2. The cDNA sequences encoding the entire extracellular regions of CD31 and M6 were amplified by PCR using the CDM8-derived plasmids TRMy005, containing the CD31 cDNA as insert (4), and H34, encoding the M6 antigen (22) as templates. An oligonucleotide complementary to the T7 promoter in the eukaryotic expression vector CDM8 was selected as universal forward primer. The reverse primers were designed to allow fusion of the extracellular domains to the human IgG1 artificial splice donor sequences in the IgG1 expression plasmid. The sequence of the reverse primer used for

CD31 amplification, containing a BglII site, was 5'-CTCGAG ATCTTTCTTCCATGGGGCAAGAATGACTCTGAC-3'; that for M6, containing a BamHI site, was 5'-CCAGAGGGGATC-CACGTGGCTGCGCACGCGGAGCGTGATGAT-3'. The PCR products were subcloned into the pGEM-T vector (Promega Corp., Madison, WI) and verified by DNA sequence analysis. After digestion of the CD31 subclone with XhoI and BglII (partial cleavage performed because of a second XhoI site in the CD31 cDNA), and of the M6 subclone with XhoI and BamHI, the extracellular domain fragments were inserted into XhoI- and BamHI-digested IgG1 expression plasmids.

For production of the chimeric molecules, the expression plasmids were cotransfected with a selectable marker plasmid, pSV2-dhfr (23) into dihydrofolate reductase-deficient CHO cells (CHO dhfr⁻) according to Chen and Okayama (24). Supernatants of transformed colonies were assayed for the presence of human IgG1 Fc fragments by ELISA using sheep anti-human Ig Abs (Boehringer Mannheim Co., Mannheim, Germany) as capture Abs, and alkaline phosphatase-conjugated goat F(ab')₂ anti-human IgG Abs (Sigma Chemical Co.) as detection Abs. Positive transformants were subcloned by limiting dilution and then passaged in increasing concentrations of methotrexate at a final level of 1 μ M. Transformants producing high levels of the chimeric molecules were expanded in serum-free Ultraculture medium (Whittaker Bioproducts, Walkersville, MD), and the recombinant proteins purified from the supernatants using a protein A-CR column (BioProcessing Ltd., Consett, UK). SDS-PAGE analysis of the purified molecules revealed bands of expected size under both reducing and nonreducing conditions.

Cells and Cell Lines. Human T cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine. Human PBMCs were isolated from peripheral blood of healthy donors using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

Human CD4⁺CD31⁻ T cell clones, TC-1, TC-2, and TC-3, were established and maintained as described elsewhere (25). Briefly, CD4⁺CD45RO⁻ T cells from healthy donors were cloned in a feeder cell-free system in 96-well round-bottom microtiter plates with OKT3-coated sheep anti-mouse IgG magnetic Dynabeads M-450 (2–5 \times 10⁴/well; Dynal, Oslo, Norway), soluble anti-Leu28 (0.5 μ g/ml), and human rIL-1 α , rIL-2 and rIL-6 (100 U/ml each; Sandoz Research Institute, Vienna, Austria) in RPMI 1640 medium supplemented with 5% pooled human serum and 2 mM L-glutamine. Clones were further propagated by a 14-d restimulation procedure using the stimuli described above. 3–4 d after each restimulation, the cultures were split and subcultured in the presence of human rIL-2 (50 U/ml) and anti-Leu28 (0.25 μ g/ml). The three clones with CD31-negative phenotype were randomly selected from different donors. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Stimulation of T Cells and T Cell Clones. PHA-activated T cells were prepared by culturing PBMCs in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine in the presence of 0.01 U/ml PHA (Wellcome, Beckenham, UK) for the times indicated.

Cloned T cells were collected on day 14 after the last restimulation with OKT3-coated beads. After removal of residual OKT3 beads, cells were activated at a concentration of 1–5 \times 10⁴ cells/well in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine in 96-well round-bottom plates for the times indicated. For stimulation via CD3, plates were precoated with OKT3 mAb at a concentration of 1 μ g/ml in PBS (50 μ l/well) over-

night at 4°C. To assess the influence of Rgs on proliferation and cytokine production, CD31Rg, M6Rg, or human IgG1 was co-immobilized on the plates at 10 µg/ml in PBS, unless otherwise indicated. After incubation, plates were washed three times with PBS and blocked for 30 min at 37°C with RPMI 1640 medium supplemented with 10% FCS. Soluble factors, i.e., PMA (60 ng/ml; Sigma Chemical Co.) plus calcium ionophore (ionomycin, 1 nM; Sigma Chemical Co.) or human rIL-2 (100 U/ml), were added to a final volume of 200 µl at the initiation of culture.

Immunofluorescence Analysis. Cells were incubated with mAbs or Rgs (20 µg/ml) in PBS containing 1% BSA and 0.02% NaN₃ for 60 min at 4°C. After washing, cells were stained for a further 30 min at 4°C with the second step reagent, FITC-conjugated sheep F(ab')₂ anti-mouse IgG + IgM Abs (An der Grub, Vienna, Austria) or PE-conjugated donkey F(ab')₂ anti-human IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. To prevent nonspecific binding of mAbs or Rgs to Fc receptors, the cells were preincubated for 30 min at 4°C with 4 mg/ml of human Ig or with 5% normal rabbit and 5% normal goat serum, respectively. To determine divalent cation and heparin sensitivity of Rg binding to T cell clones, EDTA (10 mM) or heparin (sodium salt, 12.5–50 µg/ml; Sigma Chemical Co.) was included during all incubation steps of the staining protocol. Fluorescence was analyzed on a FACScan® flow cytometer (Becton Dickinson & Co.); dead cells were excluded from the analysis by DNA staining with ethidium bromide. Viability of the cells after staining was typically >90% under all conditions tested. Cytofluorometric analysis of the T lymphocyte fraction of PBMCs was performed by electronically gating CD3⁺ cells.

Reverse Transcriptase-PCR. Total RNA was isolated from Jurkat cells and CD31⁻ Th clones by a single step method (26). First strand cDNA was prepared from 10 µg total RNA by reverse transcription using 200 U Moloney-MLV reverse transcriptase (RT; GIBCO BRL, Gaithersburg, MD) and 50 pmol oligo(dT) primer. Aliquots of RT reactions (1/20 total volume) were amplified by PCR (27) using a thermal cycler Perkin-Elmer-Cetus (Emeryville, CA) in a 50-µl final volume. The following oligonucleotides were used to amplify CD31 cDNA: F1 (sense) - 5'-AGTGAGGTTCTGAGGGTGAAGG-3', R1 (antisense) - 5'-TCACTCCGATGATAACCACTGC-3'; F2 (sense) - 5'-TCTATCCTGTCAAGTAAGGTGG-3'; and R2 (antisense) - 5'-GGCAAGAATGACTCTGACTGTC-3'. Denaturation, annealing, and extension reactions were carried out at 94°C for 45 s, 55°C for 45 s, and 72°C for 30 s, respectively, for 35 cycles. The relative amount of input cDNA was analyzed by an internal control PCR using β-actin-specific primers (28). Similar results were obtained when separate reactions were performed for the amplification of CD31 and β-actin cDNAs. Identity of amplified fragments was verified by restriction digestion.

Labeling of Cells and Precipitation. For solid phase immunoprecipitation of cell surface proteins (29), 96-well plastic plates were coated with rabbit anti-human IgG Abs (DAKO, Glostrup, Denmark) in PBS and, after washing, with CD31Rg, M6Rg, or human IgG1 at a concentration of 50 µg/ml in PBS. Cell surface proteins were biotinylated using NHS-LC-biotin (Pierce Europe, Oud-Beijerland, The Netherlands), as described previously (30). Surface-labeled cells were solubilized in lysis buffer containing 1% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 mM PMSF. The lysates were centrifuged, precleared with human IgG1 and protein A-Sepharose beads, and subjected to immunoprecipitation using the coated plates. Precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The biotinylated proteins

were visualized using streptavidin-conjugated horseradish peroxidase and the enhanced chemiluminescence detection system (Amersham International, Amersham, Bucks, UK).

To determine heparin sensitivity of Rg precipitates, Rg-coated plates were preincubated with heparin for 30 min, and heparin was included during immunoprecipitation at a concentration of 50 µg/ml.

Proliferation and Cytokine Assays. CD31⁻ Th clones were stimulated at a concentration of 10⁴ cells/well as described above for 48 and 72 h in a total volume of 200 µl in 96-well round-bottom plates. Cellular proliferation of triplicate cultures was determined by quantitating [³H]thymidine (1 µCi/well) incorporation during the last 18 h of culture. Cytokine secretion by T cell clones was analyzed in supernatants of the stimulation assays after 48 h of culture. Concentrations of IL-4, IFN-γ, and TNF-α were determined by antigen-capture ELISA, as described previously (12, 31).

Results

Expression of a Heterophilic Ligand for CD31 on Activated T Lymphocytes. To determine whether human T cells express a heterophilic ligand for CD31, we first examined the reactivity of CD31Rg with several human T cell lines. A comparison of the binding activities of CD31Rg and CD31 mAbs, as assessed by indirect immunofluorescence and flow cytometric analysis, revealed that T cell lines with both CD31⁺ (Jurkat, Molt-4) and CD31⁻ phenotype (HUT-78, HPB-ALL) specifically bound CD31Rg, but not an analogous control fusion protein (M6Rg) or human IgG1 (data not shown). Thus, this initial experiment indicated that human T cells might interact with CD31Rg in a homophilic and/or heterophilic way.

It has been shown that CD31 is expressed on ~50% of human peripheral blood T lymphocytes, and that this bimodal expression remains virtually unchanged upon cellular activation (5, 19). When we examined the ability of CD31Rg to interact with freshly isolated human peripheral blood T lymphocytes, both the CD31⁺ and CD31⁻ subsets of T lymphocytes failed to stain with CD31Rg. However, after stimulation with PHA in the presence of accessory cells, 90 ± 3% (n = 6) of the T blasts specifically bound the chimeric protein, but showed no significant reactivity with M6Rg or human IgG1. CD31Rg binding reached a peak level as early as 18 h after activation, maintained it for a further 48 h, and declined thereafter (Fig. 1 A). In contrast, the expression of the CD31 molecule, as determined by CD31 mAb staining, was restricted to 61 ± 4% of the T lymphocytes in their resting state and decreased to 54 ± 2% of cells at the time point of maximal CD31Rg binding. CD31 expression was slightly increased at day 4 of activation when CD31Rg staining had returned almost to the level of resting cells (Fig. 1 B). These differential binding patterns of CD31 mAbs and CD31Rg indicate that the chimeric fusion protein identifies a heterophilic CD31 ligand that is expressed by at least 36% of human T lymphocytes and is temporally regulated during T cell activation. We therefore designate this T cell surface molecule recognized by CD31Rg as the T cell CD31 ligand (T-CD31L).

Expression of a Heterophilic Ligand for CD31 on Activated CD31⁻ T Cell Clones. To characterize T-CD31L with-

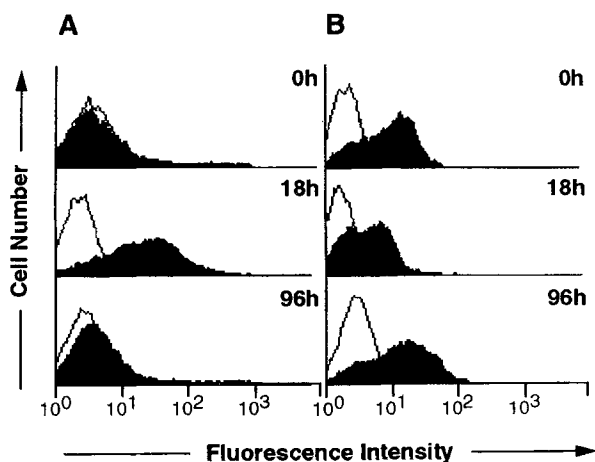


Figure 1. Reactivity of CD31Rg and CD31 mAb with activated human peripheral blood T lymphocytes. Human PBMCs were activated with PHA (0.01 U/ml) for the times indicated, and the binding of CD31Rg or CD31 mAb to T cells was analyzed by indirect immunofluorescence and flow cytometry. (A) Reactivity of CD31Rg (shaded area) or control M6Rg (open area). (B) Reactivity of CD31 mAb 7E4 (shaded area) or isotype-matched control mAb (open area). Identical staining profiles were obtained with CD31 mAb 5E6. The result is a representative of six independent experiments.

out any partial influence of a putative CD31 homophilic interaction, we established a panel of human T cell clones with CD31⁻ phenotype (see Materials and Methods). Three clones derived from different donors (termed TC-1, TC-2, and TC-3) were chosen for all subsequent experiments. These clones were classified as CD3⁺CD4⁺CD8⁻CD31⁻ using indirect immunofluorescence and FACS[®] analysis (Becton Dickinson & Co.; data not shown). A kinetic analysis of Rg reactivity by the clones revealed a specific binding of CD31Rg that was strictly dependent on the activation state of the cells, as seen earlier with peripheral T lymphocytes. CD31Rg binding reached peak levels 18 h after restimulation via immobilized CD3 mAb OKT3 and IL-2 (>90% positive cells) and decreased gradually 3 d after activation (Fig. 2). This apparent upregulation of a CD31Rg-binding molecule was independent of the stimulus used, since the same results were obtained when the clones were restimulated via immobilized CD3 mAb alone or in the presence of PMA plus calcium ionophore (data not shown). However, under all the restimulation conditions assayed, no binding of CD31 mAbs could be detected (data not shown), therefore distinguishing these clones as potential candidates for the study of the heterophilic interaction of CD31 and T-CD31L on a homogenous CD31⁻ T cell population.

To conclusively prove the CD31⁻ phenotype of the T cell clones, we had to exclude the possibility that CD31Rg recognized an isoform or posttranslational modification variant of CD31 undetectable by the CD31 mAbs used. To address this issue, we analyzed CD31 expression at the RNA level using CD31-specific primers. RT-PCR analysis of RNA prepared from the resting as well as the previously activated

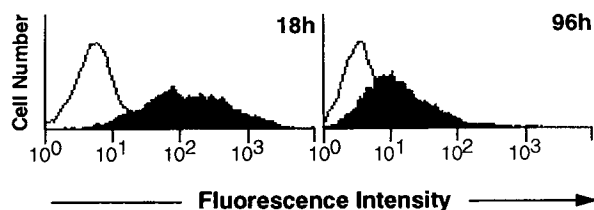


Figure 2. Expression of a heterophilic counterreceptor for CD31 on activated human CD31⁻ T cell clones. T cell clone TC-1 was restimulated via immobilized CD3 mAb OKT-3 plus IL-2 for the times indicated and then analyzed for reactivity with CD31Rg by indirect immunofluorescence and flow cytometry. (Open areas) Background levels of fluorescence obtained with control M6Rg. The data are representative of five experiments. Similar results were obtained with T cell clones TC-2 and TC-3.

T cell clones demonstrated the absence of CD31 transcripts in these cells, whereas CD31 mRNA was easily detected in the CD31⁺ T cell line Jurkat (Fig. 3). These results were obtained whether RNA was isolated 1, 2, or 14 d after restimulation of the T cell clones.

Effect of Divalent Cations and Heparin on CD31Rg Binding to T Cells. Previous reports have shown that the CD31-mediated heterophilic aggregation of mouse L cells is divalent cation dependent and involves heparin or chondroitin sulfate residues on the surface of adjacent cells (9–11). We therefore investigated whether divalent cations or heparin could modify the binding of CD31Rg to activated CD31⁻ T cell clones. Preincubation of CD31Rg with heparin (12.5–50 μg/ml) completely abolished the binding of the chimeric construct to the clones, resembling its effect in the L cell aggregation system (Fig. 4 A). However, Ca²⁺ and Mg²⁺ ions were not essential for CD31Rg binding, insofar as EDTA rather enhanced the mean fluorescence intensity of CD31Rg-stained cells by 37 ± 6% (n = 3) as compared with divalent cation-containing medium (Fig. 4 A). Reactivity of a positive control fusion protein, CD2Rg, remained virtually unchanged under all conditions tested

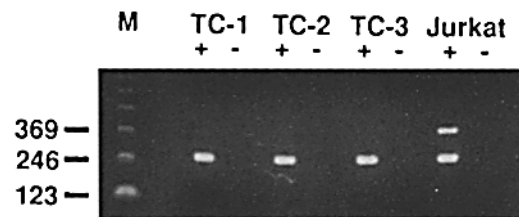


Figure 3. CD31⁻ T cell clones express no CD31 mRNA. RT-PCR analysis of RNA from human CD31⁻ T cell clones TC-1, TC-2, and TC-3 as well as the CD31⁺ T cell line Jurkat was performed using the CD31-specific oligonucleotides, F1 and R1. The same findings were obtained with a different set of CD31-specific primers, F2 and R2 (see Materials and Methods). Equal aliquots of PCR reactions were electrophoresed on a 2% agarose gel containing ethidium bromide (+). Negative control reactions were performed without RT (-). The relative amount of input cDNA was standardized by an internal control PCR using β-actin-specific primers. The expected size of the CD31 and β-actin PCR products are 369 and 245 bp, respectively. (Lane M) A 123-bp DNA ladder as size standard. The data are representative of three experiments.

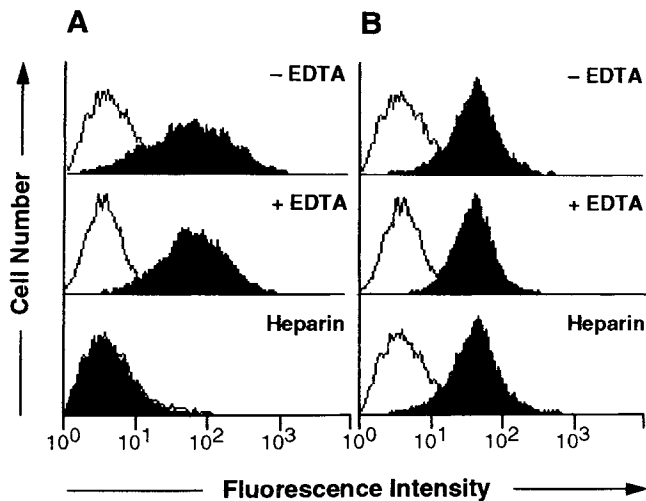


Figure 4. Effect of divalent cations and heparin on CD31Rg binding to human CD31⁻ T cell clone TC-1. Stimulation was performed as indicated in the legend to Fig. 2. The cells were analyzed 48 h after activation for reactivity with CD31Rg (A) or CD2Rg (B) by indirect immunofluorescence and flow cytometry. Staining with Rgs was performed in PBS containing either 1 mM Ca²⁺ and 1 mM Mg²⁺ (-EDTA), 10 mM EDTA (+EDTA), or 12.5 μg/ml heparin. (Open areas) Background levels of fluorescence obtained with control M6Rg. The data are representative of three independent experiments.

(Fig. 4 B), demonstrating that these effects were CD31Rg specific.

Immunoprecipitation of Cell Surface Protein Binding to CD31Rg. To characterize the molecular nature of the T cell surface molecule involved in CD31Rg binding, TC-1 cells were surface biotinylated, and the lysates were subjected to precipitation with CD31Rg. Analysis of the precipitates by SDS-PAGE showed a prominent band of ~120 kD, that was selectively present in the lysates of previously activated, but not of resting TC-1 cells (Fig. 5 A, lanes 1 and 3). This protein was identical in size under both nonre-

ducing and reducing conditions (Fig. 5 A, lanes 3 and 3'), and was not observed in precipitates of control M6Rg or human IgG1 (Fig. 5 A, lanes 4 and 4'). Affinity isolation experiments using T cell clones TC-2 and TC-3, as well as the CD31⁻ T cell lines HUT-78 and HPB-ALL revealed the same specific 120-kD band (Fig. 5 B, lanes 1-4, and data not shown). Precipitation of the 120-kD protein from the T cell clones and T cell lines by CD31Rg was completely abolished when the immunoisolation was performed in the presence of heparin (Fig. 5 C, lanes 1-4). This finding supports the involvement of GAGs in CD31Rg reactivity with different cell types of T cell origin.

CD31Rg Inhibits Activation of Human CD31⁻ T Cell Clones. CD31 has been shown to represent a cell surface marker that allows CD4⁺ T cells to be subdivided into those that exert the suppressor inducer (CD31⁺), and the helper (CD31⁻) function for B cell IgG synthesis (19). This observation, together with our finding that expression of T-CD31L is restricted to T cells at certain stages of activation, prompted us to examine whether T-CD31L/CD31 might represent a novel accessory system of T cells. We first assessed the influence of CD31Rg on activation-induced IL-2 production by the human CD31⁻ T cell line HUT-78. In the presence of immobilized CD3 mAb, HUT-78 cells produced moderate levels of IL-2 (32), which significantly increased upon costimulation with CD28 mAb. Addition of CD31Rg to CD3-stimulated cultures did not augment IL-2 accumulation, indicating that the fusion protein could not provide a costimulatory signal under these conditions. In contrast, when HUT-78 cells were stimulated maximally via immobilized CD3 plus CD28 mAbs in the presence of soluble CD31Rg, a significant inhibition (~60%) of IL-2 production was observed as compared with cultures stimulated with mAbs alone or in the presence of Rg controls (data not shown). These experiments suggested that engagement of T-CD31L by CD31Rg may be involved in the down-regulation of T cell responses after initial activation.

To analyze the inhibitory potential of CD31Rg in more

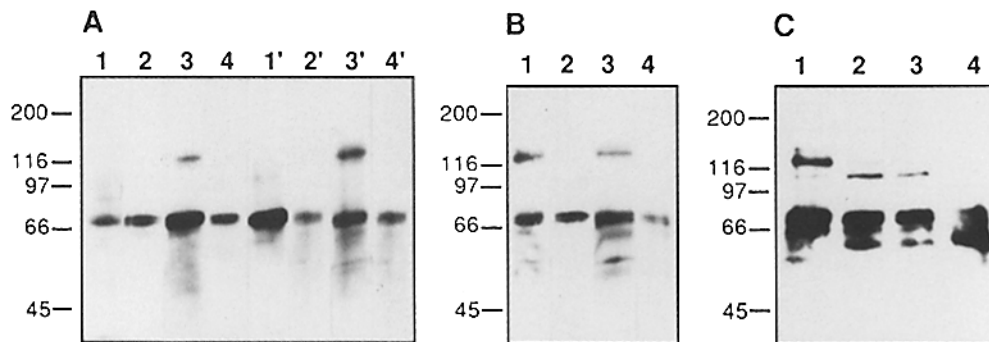


Figure 5. Biochemical characterization of the CD31Rg-binding protein. Precipitates obtained from lysates of biotin surface-labeled TC-1 cells and human T cell lines were analyzed by SDS-PAGE under nonreducing conditions. After transfer to a nitrocellulose membrane, biotin-labeled proteins were visualized using a streptavidin-biotinylated horseradish peroxidase complex and enhanced chemiluminescence detection system. (A) Immunoprecipitation with resting (lanes 1 and 2) and previously activated (lanes 3 and 4) TC-1 cells using CD31Rg (lanes 1 and 3) or control M6Rg (lanes 2 and 4). These samples were also analyzed under reducing conditions (lanes 1'-4'). (B) Precipitation with HUT-78 (lanes 1 and 2) and HPB-ALL cells (lanes 3 and 4) using CD31Rg (lanes 1 and 3) and control M6Rg (lanes 2 and 4). (C) Precipitates obtained from activated TC-1 cells using CD31Rg (lane 1) and control M6Rg (lane 4) in the absence, or CD31Rg (lane 2) and M6Rg (lane 3) in the presence of 50 μg/ml heparin. Biotinylated molecular mass standards are indicated in kilodaltons. The results are representative of three independent experiments for A, B, and C, respectively.

detail, we examined its influence on proliferative capacity and cytokine production of the CD4⁺CD31⁻ T cell clones TC-1, TC-2, and TC-3. The clones were restimulated via immobilized CD3 mAb in the presence or absence of CD31Rg, and [³H]thymidine incorporation was determined during the last 18 h of 48- and 72-h cultures. The results obtained are shown in Fig. 6 A with clone TC-1 as a representative. The proliferative response of the cells was reduced by 75 ± 2% (*n* = 4) when CD31Rg (10 µg/ml) was coimmobilized on the plates, whereas no inhibitory effect was observed after coimmobilization of control M6Rg or human IgG1. Similar results were obtained when CD31Rg was added in soluble form, although the inhibitory effect was less pronounced (data not shown). A titration experiment revealed a significant inhibition of [³H]thymidine incorporation with CD31Rg immobilized at 5 µg/ml, and an almost complete abrogation of the proliferative response at 20 µg/ml (Fig. 6 B). However, coimmobilization of CD31Rg had no influence on proliferation when we added IL-2 to these cultures. Furthermore, although TC-1 cells proliferated only modestly upon stimulation with PMA and calcium ionophore, this response could also not be blocked by immobilized CD31Rg (Fig. 6 A). Addition of soluble CD28 mAb to CD3-stimulated cultures neither augmented the CD3-induced response nor reversed the inhibitory effect of CD31Rg (data not shown). This apparent unresponsiveness to CD28 stimulation might result from the pro-

longed culture of the T cell clones in the presence of CD28 mAbs (see Materials and Methods), resulting in a desensitization of their CD28-signaling pathway (33).

Next, we evaluated the profile of cytokine release from the T clones in response to immobilized CD3 mAb or PMA plus calcium ionophore. All three clones secreted high levels of IL-4, low levels of IFN-γ, and no or minimum amounts of IL-2, a pattern that resembles a Th2/0 cell type (34). As in the proliferation assay, addition of CD28 mAb to CD3-stimulated cultures had no significant effect.

To assess the influence of CD31Rg on the cytokine secretion capacity of the clones, supernatants were collected 48 h after restimulation and analyzed for the presence of IL-4, IFN-γ, and TNF-α. Immobilized CD31Rg (10 µg/ml) specifically inhibited the release of all three cytokines when the cells were activated via immobilized CD3 mAb. IL-4 secretion was reduced by 91 ± 2% (*n* = 4), whereas IFN-γ and TNF-α release were decreased by 92 ± 7% and 88 ± 7%, respectively (Fig. 7). The inhibition was dose dependent, and complete downregulation of cytokine production to the basal levels of unstimulated cultures was observed when CD31Rg was immobilized at 20 µg/ml (data not shown). In contrast to its ability to completely abrogate the inhibitory effect of CD31Rg in the proliferation assays, IL-2 could only partially reverse the inhibition of cytokine release (Fig. 7). Thus, the inhibitory effect of CD31Rg on

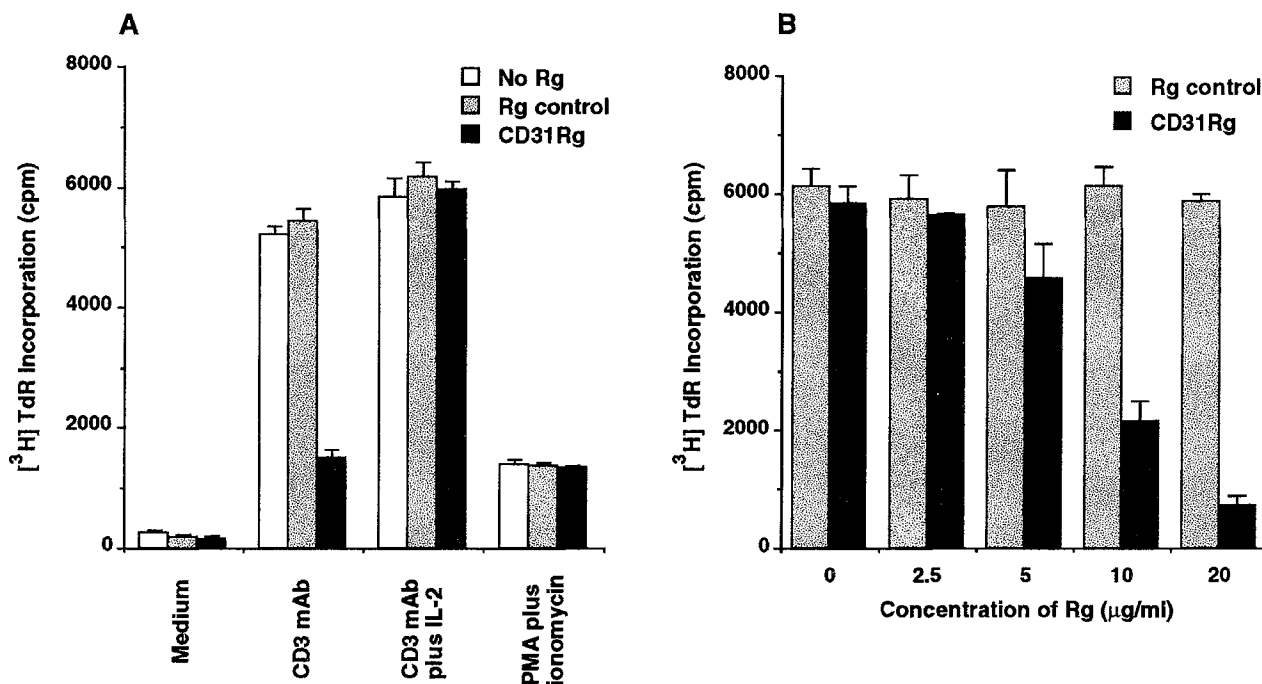


Figure 6. Effect of CD31Rg on the proliferative response of human CD4⁺CD31⁻ T cell clone TC-1. (A) Cells (10⁴) were cultured in the presence of CD3 mAb OKT3 (1 µg/ml) coimmobilized with CD31Rg or a control Rg (10 µg/ml) alone or in combination with IL-2 (100 U/ml). In addition, TC-1 cells were also restimulated with PMA (0.1 nM) plus the calcium ionophore ionomycin (1 nM) in the presence of immobilized CD31Rg or control Rg. The data presented are representative of four independent experiments. (B) TC-1 cells were cultured in the presence of CD3 mAb OKT3 (1 µg/ml) coimmobilized with CD31Rg or control Rg at the concentrations indicated. Proliferation was determined at 48 h. Results are expressed as mean cpm ± SE of triplicate determinations.

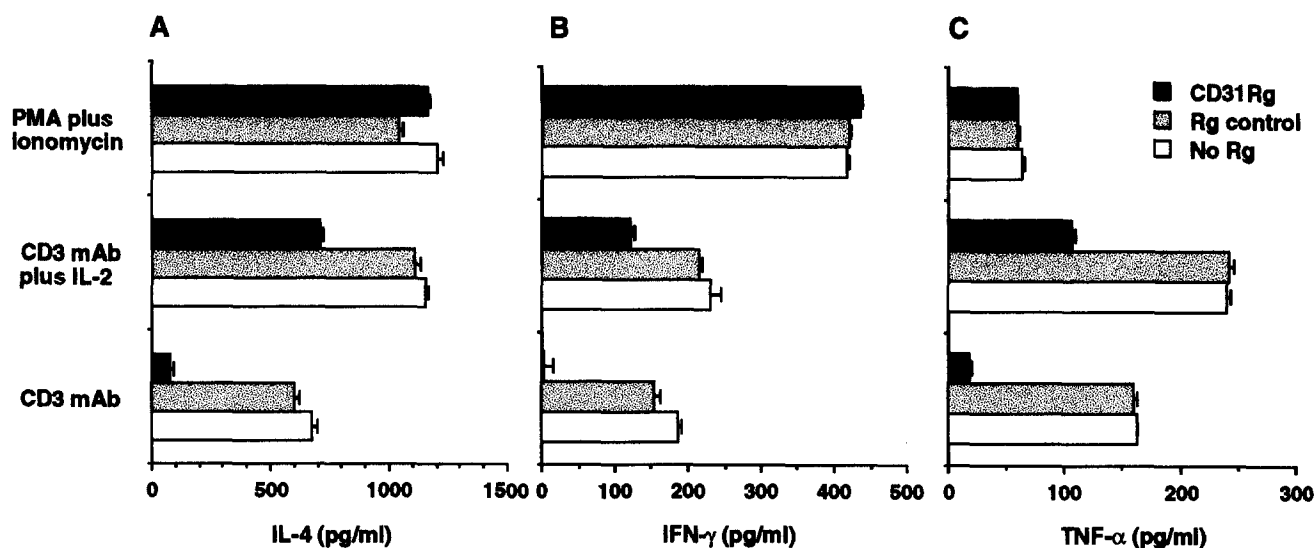


Figure 7. Effect of CD31Rg on the cytokine secretion of human CD4⁺CD31⁻ T cell clones. TC-1 cells were cultured as described in Fig. 6 A. After 48 h of restimulation, supernatants were collected and IL-4 (A), IFN- γ (B), or TNF- α (C) concentrations were measured by ELISA. Data are expressed as mean \pm SE of duplicate determinations. Note that different scales are used for each of the three cytokines. The results are representative of four independent experiments. The same findings were obtained in experiments performed with the clones TC-2 and TC-3.

cytokine release seems to be not just a consequence of decreased proliferation rate and reduced cell number. Similar to the proliferation assays, restimulation of TC-1 clones with PMA and calcium ionophore completely bypassed the blocking effect of CD31Rg (Fig. 7).

Discussion

In recent years, several reports have demonstrated that CD31 can be considered as an adhesion molecule with both homophilic and heterophilic binding properties (9–11, 35, 36). The interaction mechanisms of leukocyte CD31, in particular, have been studied primarily with regard to the process of transendothelial migration (15, 18, 37, 38); however, very little is known about the molecular nature of a putative heterophilic counterreceptor(s) of CD31 on leukocytes, and its function in their internal communication. Using a soluble CD31Rg containing the entire extracellular domain of CD31, we demonstrate here that activated human T cells express a heterophilic CD31 counterligand with potential accessory function in T lymphocyte responses.

At 18 h after activation of T lymphocytes, when not >54% of T cells expressed CD31, a ligand for CD31Rg was expressed on the surface of almost all of these cells, and completely downregulated 4–5 d after activation. The heterophilic nature of the interaction of CD31Rg with human T cells was corroborated by the generation of human CD31⁻ T cell clones that also bound CD31Rg upon activation, but did not display either reactivity with CD31 mAbs, or expression of CD31 mRNA.

We designated this heterophilic CD31 ligand expressed by activated T cells T-CD31L because its features distinguish it from all other heterophilic ligands so far identified. First, in the mouse L cell aggregation assay, CD31-mediated

heterophilic adhesion has been shown to depend on divalent cations and to be perturbed by restricted classes of GAGs, such as heparin and heparan sulfate. Accordingly, it has been proposed that CD31, via a consensus heparin-binding motif in the second Ig-like domain, could bind to GAGs in the extracellular matrix, or to a GAG-containing counterreceptor on the surface of adjacent cells (9, 10). The binding properties of CD31Rg to T-CD31L only partially concur with these observations. Heparin specifically abolished the reactivity of CD31Rg with CD31⁻ T cell lines and activated T cell clones, yet the interaction did not require the presence of Ca²⁺ and Mg²⁺ ions. By contrast, removal of divalent cations reduced the reactivity of CD31Rg with human CD31⁻ fibroblastoid cell lines by 30–70% (data not shown). Second, while this manuscript was in preparation, $\alpha_v\beta_3$ integrin (CD51/CD61 complex) has been reported to serve as a ligand for a truncated recombinant form of CD31 containing the three NH₂-terminal Ig-like domains of the molecule (39). Contrary to the CD31Rg–T-CD31L interaction, binding of CD31 to $\alpha_v\beta_3$ has been demonstrated to be divalent cation dependent but insensitive to heparin. The difference between the two CD31 counterreceptors, T-CD31L and $\alpha_v\beta_3$ integrin, is further emphasized by our biochemical studies: CD31Rg precipitated a single chain protein of 120 kD from surface-labeled, activated Th clones, which could specifically be displaced by addition of heparin (Fig. 5). Moreover, our T cell clones showed no significant reactivity with CD51 and CD61 mAbs (data not shown).

Taken together, these observations suggest that T-CD31L represents an additional counterreceptor for CD31, which might be associated with a similar GAG-containing carbohydrate structure as the ligand described in the L cell system. Thus, similar to other members of the Ig superfamily,

CD31 seems to be capable of interacting with a multiplicity of counterligands on different cell types. Alternative splicing of the cytoplasmic domain of CD31 has been reported to result in a change of the configuration of the extracellular, ligand-binding domain of the molecule (40–42), and intracellular phosphorylation of CD31 might as well modulate its adhesive properties (43). It is conceivable to speculate therefore, that different cytoplasmic variant forms of CD31 each interact preferentially with CD31 in a homophilic fashion or with one of the heterophilic counterligands described so far, thereby offering the opportunity to regulate specific cell–cell interactions.

The diversity of, possibly cell type–specific, counter-receptors for CD31 might also account for the different physiological functions that have been attributed to this molecule (see Introduction). The binding of CD31 to $\alpha_v\beta_3$, for example, has been implicated in leukocyte–endothelial interaction, indicating an involvement of this adhesion pair in the transendothelial migration process (39). On the other hand, we here provide evidence that T-CD31L represents a novel accessory molecule of T lymphocytes involved in the downregulation of effector Th cell responses. Activation of human CD31⁺ Th clones in the presence of cross-linked or soluble CD31Rg completely abrogated the proliferative response and production of effector cytokines (IL-4, IFN- γ , and TNF- α) when the cells were maximally stimulated with a CD3 mAb. CD31Rg seems to interfere primarily with the autocrine and paracrine cytokine production of Th cells, since exogenous IL-2 could reverse the inhibitory effect of CD31Rg on proliferation, but only partially restore the cytokine-producing capacity of the clones (compare Figs. 6 and 7). Therefore, the inhibitory effect of CD31Rg on the proliferative response might be due merely to the downmodulation of autocrine IL-2 production. A complete reversal of the CD31Rg-mediated inhibition could only be accomplished when the TCR signaling was bypassed by stimulation with PMA and calcium ionophore, suggesting that CD31Rg interferes with a step upstream of signal-induced calcium influx and protein kinase C activation.

Although our results do not rule out the possibility that CD31Rg exerts a different, maybe opposite effect on the responses of naive/memory T cells, preliminary data suggest that the fusion protein can also inhibit proliferation and cytokine production of freshly isolated human T lymphocytes (data not shown). Furthermore, the blocking effect of CD31Rg observed in our functional assays is supported by a recent report by Zehnder et al. (20), who

demonstrated that a peptide derived from CD31 could specifically inhibit T lymphocyte responses in primary MLRs. However, whereas a direct adhesive mechanism involving CD31 has not been established in these studies, our phenotypic and biochemical results are consistent with a heterophilic interaction of CD31Rg with Th cells that may result in the transmission of a negative signal inside the cell. Alternatively, the binding of CD31Rg might upregulate or activate a negative regulatory molecule on the surface of the T clones, which then interacts with a putative counter-receptor on apposing T cells. A third explanation of the data presented here is that CD31Rg could compete with the interaction of a surface protein with a soluble factor or a counterligand on adjacent cells, thereby preventing the transduction of a positive signal.

Although a number of cell surface receptors are implicated in the costimulation or augmentation of T cell activation, molecules whose function is to dampen or downregulate T cell reactivity have not been extensively investigated. The most well-characterized accessory molecule of T lymphocytes with a supposed negative regulatory function is CTLA-4, a cell surface protein of the Ig superfamily induced during T cell activation (44). Anti-CTLA-4 mAbs can block the proliferative response and cytokine production of T lymphocytes (45, 46) and trigger antigen-specific clonal deletion of previously activated T cells (47). The inhibition of Th cell responses by CD31Rg is reminiscent of the downregulatory effect of CTLA-4 mAbs. However, unlike CTLA-4 engagement by mAbs, interaction of CD31Rg with T-CD31L does not appear to result in enhanced apoptosis of previously activated Th clones, as assessed by propidium iodide staining and FACS[®] analysis at different time points after activation (data not shown). In analogy to the multiplicity of positive regulatory costimulators of T cell activation (48, 49), it might be hypothesized that several functionally different cell surface molecules participate in the termination of a prevailing T cell response, thereby regulating the strength and specifics of the particular immune reaction. Alternatively, the CD31/T-CD31L adhesion pair might play a more specialized role in the physiology of CD31⁺ T lymphocytes. Since CD31 has been demonstrated to define cells of the suppressor lineage (19), its heterophilic interaction with T-CD31L on the surface of CD31⁺ Th cells might result directly in the generation of suppressor signals. The elucidation of the definite role of T-CD31L in T cell immunity must await the molecular cloning of this molecule and a more detailed analysis of its function in the responses of CD31⁺ and CD31⁺ T lymphocytes.

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